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A deep divergence time between sister species of *Eidolon* (Pteropodidae) with evidence for widespread panmixia

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The pteropodid fruit bat genus *Eidolon* is comprised of two extant species: *E. dupreanum* on Madagascar and *E. helvum* on the African mainland and offshore islands. Recent population genetic studies of *E. helvum* indicate widespread panmixia across the continent, although island populations off western Africa show genetic structure. Little is known about the genetic connectivity of *E. dupreanum* or the divergence time between these two sister species. We examine sequence data for one mitochondrial (cyt-*b*) and three nuclear regions (*β-fib*, *RAG1*, and *RAG2*) to assess population genetic structure within *E. dupreanum* and divergence between the two *Eidolon* spp. In addition, we characterize the demographic history of both taxa using coalescent-based methods. We find little evidence for population structure within *E. dupreanum*, and suggest that this reflects dispersal based on seasonal fruit availability and a preference for roosting sites in exposed rock outcrops. However, despite apparent panmixia in both *Eidolon* spp. and large dispersal distances reported in previous studies for *E. helvum*, these two taxa diverged in the mid-to-late Miocene. Both species are also characterized by population expansion and young, Pleistocene clade ages, although slower population growth in *E. dupreanum* is likely explained by its divergence via colonization from the mainland. Finally, we discuss the implications of population connectivity in *E. dupreanum* in the context of its potential role as a reservoir host for pathogens capable of infecting humans.

Key words: phylogeography, divergence time, Africa, Madagascar, *Eidolon*

INTRODUCTION

The bat genus *Eidolon* (family Pteropodidae) contains two species: *E. helvum* Kerr, 1792 — the largest and most common fruit bat in sub-Saharan Africa, certain neighboring African offshore islands, and the Arabian Peninsula — and *E. dupreanum* Pollen, 1866, endemic to Madagascar (Bergmans, 1990; Simmons, 2005). The day roost sites of *E. helvum* can contain notably large numbers of individuals, and are typically found in trees within forest, savannah, or urban areas (Nowak and Roland, 1999; Sørensen and Halberg, 2001). Continental populations of *E. helvum* are seasonally migratory, and while extremely long distance movements have been recorded (Richter and Cumming, 2008), it is unknown whether migration is directed or represents nomadic movements following seasonal changes in resource availability.

By contrast, *E. dupreanum* rarely roosts in trees. Instead, these bats preferentially aggregate in small colonies within rock crevasses and caves (MacKinnon *et al*., 2003; Racey *et al*., 2009; Goodman, 2011). Recent research on *E. dupreanum* cave roosts indicates that they navigate in and out of these structures with what appears to be an incipient form of echolocation (Schoeman and Goodman, 2012); this is a roosting and behavioral niche

generally not utilized by its sister species *E. helvum* (Nowak and Roland, 1999). *Eidolon dupreanum* is also an important seed disperser on Madagascar and plays a critical role in tree regeneration (Picot *et al*., 2007; Ratrimomanarivo, 2007).

Eidolon dupreanum is one of the three species of endemic pteropodid bats on Madagascar, all of which are subject to considerable hunting pressure (Jenkins and Racey, 2008; Goodman, 2011). Phylogeographic studies conducted on two of these species, *Pteropus rufus* E. Geoffroy, 1803 and *Rousettus madagascariensis* G. Grandidier, 1929, reveal little evidence for intraspecific population structure across the island and suggest genetic panmixia in both taxa (Goodman *et al*., 2010; Chan *et al*., 2011). A similar pattern has been found on Madagascar in widely-foraging insectivorous bats of the family Molossidae, such as *Mops leucostigma* G. M. Allen, 1918 (Ratrimomanarivo *et al*., 2008) and *Mormopterus jugularis* Peters, 1865 (Ratrimomanarivo *et al*., 2009). These patterns contrast with the geographically structured genetic variation found within other insectivorous Malagasy bats, including *Triaenops furculus* Trouessart, 1906 (Russell *et al*., 2007), *Myzopoda aurita* Milne-Edwards and A. Grandidier, 1878 (Russell *et al*., 2008), *Myotis goudoti* A. Smith, 1834 (Weyeneth *et al*., 2011), and *Chaerephon atsinanana* Goodman, Buccas, Naidoo, Ratrimomanarivo, Taylor and Lamb, 2010 (Lamb *et al*., 2012). If body size, ecology, and phylogenetic history are correlated with population structure (Carmichael *et al*., 2007), we predict that *E. dupreanum* is characterized by high population connectivity, as found among other pteropodid bats.

Population structure in bats is hypothesized to be driven, in part, by a combination of social and feeding behaviors (Barclay, 1991; Rivers *et al*., 2005), body size (Isaac *et al*., 2005), and dispersal capabilities (Petit and Mayer, 2000; Russell *et al*., 2005). Previous work on *E. helvum* suggests that African continental populations are characterized by high haplotype diversity, but with no geographic structure among them, representing genetic connectivity across a geographical scale greater than previously recorded for any mammal species (Peel *et al*., 2013). A concordant study utilizing stable isotopes also demonstrates that *E. helvum* is wide-ranging across geographically distinct regions of southern Africa (Ossa *et al*., 2012). In contrast, marked genetic structure occurs in populations of this species occurring on offshore islands in the Gulf of Guinea (Juste *et al*., 2000; Peel *et al*., 2013). High vagility of *Eidolon* in general, with the exception of certain island populations, may explain its low species-level diversity (Juste *et al*., 2000).

It is unclear if extensive gene flow characterizes *E. dupreanum*, as would be expected given the patterns in *E. helvum* and in the other two Malagasy pteropodid species. To date, nothing is known about the connectivity of *E. dupreanum* populations based on either inference from phylogeographic studies or direct observations through tracking studies. Importantly, the structure of *Eidolon* populations may be directly associated with transmission patterns in certain zoonoses. *Eidolon dupreanum* and *E. helvum* are both known reservoirs of potential pathogens (e.g., Iehlé *et al*., 2007; Drexler *et al*., 2012; Hayman *et al*., 2012; Peel *et al*., 2013). Humans and fruit bats have complex epidemiological interactions in Africa and on Madagascar, with exchange of blood, urine, and saliva via the bush meat trade. In addition, these bats feed on fruits and roost in sites in close proximity to humans. In concert, these factors underscore the need for continued studies on the ecology and connectivity of *Eidolon* populations.

We use DNA sequence data to characterize the population genetic structure of *E. dupreanum* across Madagascar, and compare the results with previously reported patterns in its sister species, *E. helvum*, across sub-Saharan Africa. In addition, we estimate the clade age of each species and their divergence time to provide greater insight into their shared and unique evolutionary histories.

Materials and Methods

Sampling

Tissues were collected throughout the respective ranges of *E. dupreanum* (n = 80) and *E. helvum* (n = 37) using varied sampling techniques (Fig. 1, Appendix). For *E. dupreanum*, we obtained 39 wing punch samples and 41 muscle samples. These tissues were preserved in 0.5% EDTA buffer, and any voucher specimens collected were deposited in either the Field Museum of Natural History (Chicago, FMNH) or the Université d'Antananarivo, Département de Biologie Animale (Antananarivo, UADBA). For *E. helvum*, we obtained 37 wing punch samples, and preserved them in 70% alcohol (with the exception of one museum specimen, T181, preserved in 10% buffered formalin). Fieldwork on *E. helvum* and *E. dupreanum* was conducted under permits granted by national and local authorities. Research involving live animals followed guidelines for the capture, handling, and care of mammals approved by the American Society of Mammalogists (Sikes *et al*., 2011).

DNA Extraction, Amplification, and Sequencing

We extracted whole genomic DNA from samples with the DNeasy Tissue Kit (Qiagen). For all samples, including outgroups, we first targeted the mitochondrial cytochrome-*b* gene (cyt-*b*). Three nuclear regions (intron 7 of the nuclear β-fibrinogen gene, *β-fib*, and portions of the recombination activating genes 1 and 2, *RAG1* and *RAG2*) were targeted for only a subset of *Eidolon* samples from each locality, as we expected intrapopulation diversity to be low at these loci. Amplification of loci proceeded using the following primers: L14724 and H15915 for cyt-*b* (Irwin *et al*., 1991); bfib-mammU and b17-mammL for *β-fib* (Matocq *et al*., 2007); RAG1F1705, RAG1R2864, RAG2F220, and RAG2R995 for the single exons of *RAG1* and *RAG2* (Teeling *et al*., 2005).

We conducted PCRs in 25 μL reactions with 1 × buffer, 2.0 mM $MgCl_2$ (1.6 mM for *β-fib*), 0.2 mM dNTPs, 0.4 μM of the locus-specific primers, 0.5 U (0.625 U for cyt-*b*) of *Taq* polymerase and 1 μL template DNA. Initial denaturation proceeded at 94ºC for 3 min, followed by 35 cycles of 45 sec at 94ºC, 45 sec at 52ºC (cyt-*b*), 56ºC (*β-fib*), or 60ºC (*RAG1* and *RAG2*), and 75 sec at 72ºC, and a final extension at 72ºC for eight min.

PCR products were cleaned in preparation for cyclosequencing using ExoSAP-IT (USB Products): 5 μL of PCR product was incubated with 1.6 μL of sterile H_2O and 0.4 μL of ExoSAP-IT at 37ºC for 15 min, followed by 80ºC for 15 min. We sequenced each locus in complementary directions with their respective PCR primers; each reaction included 0.75 μL of purified PCR product, 0.75 μL of 5 × buffer, 0.5 μL of primer, and 0.2 μL of BigDye v3 in a total volume of 5 μL. Final sequence solutions were electrophoresed on an ABI 3730xl capillary sequencer.

We checked, trimmed, and assembled electropherograms into contigs using Sequencher v4.8 (GeneCodes, Ann Arbor, Michigan). We aligned consensus sequences with MacClade

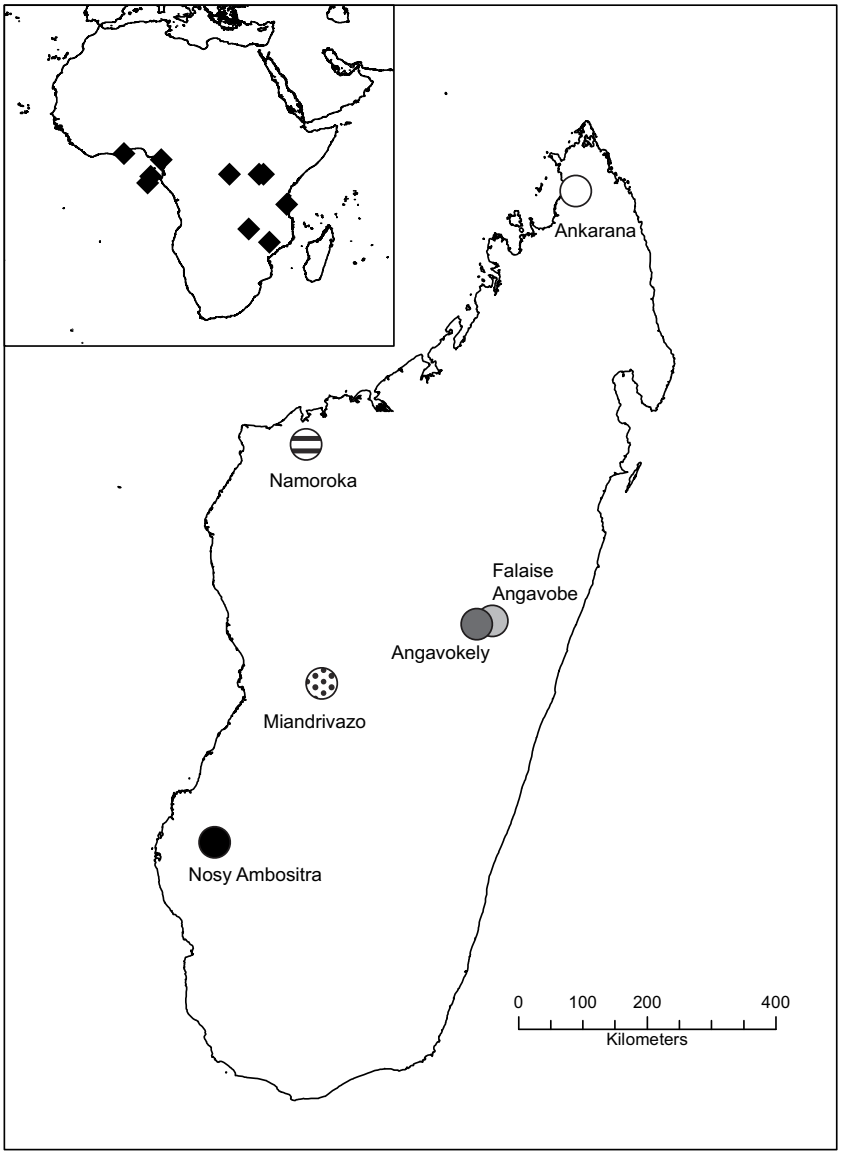

FIG. 1. Map of sampling localities for *E. helvum* across sub-Saharan Africa (inset) and *E. dupreanum* on Madagascar

v4.08 (Maddison and Maddison, 2008). For the cyt-*b* alignment, we also included previously sequenced data for two outgroup species in the genus *Pteropus* (*P. seychellensis comorensis* Nicoll, 1880, GenBank accession number JF327290.1; *P. rufus*, JF327321.1) and two in the genus *Rousettus* (*R. madagascariensis*, GU228603.1; *R. obliviosus* Kock, 1978, GU228742.1).

Phylogenetic Analysis

Based on the proximity of putative *RAG1* and *RAG2* genes in the *Pteropus* draft genome (Lindblad-Toh *et al*., 2011) and their proximity in the mouse genome (Oettinger *et al*., 1990), we concatenated the two loci using Phyutility (Smith and Dunn, 2008). The cyt-*b*, *β-fib*, and concatenated *RAG1-RAG2* alignments were reduced to unique haplotypes using the Biopython script 'sequence_cleaner.py' (Cock *et al*., 2009).

We constructed haplotype networks for *E. dupreanum* at cyt-*b*, and for both species of *Eidolon* at both *β-fib* and *RAG1-RAG2*, using TCS with a connection limit of 90% and allowing for ambiguous characters (Clement *et al*., 2000). Because we found high sequence divergence between *E. dupreanum* and *E. helvum* at cyt-*b*, and because previous research on cyt-*b* in *E. helvum* on continental Africa indicated little geographic clustering of haplotypes (Peel *et al*., 2013), we did not construct haplotype networks for *E. helvum* for this locus. The geographic localities of haplotypes for *E. dupreanum* were visualized on each network to examine correspondence between genetic diversity and geography.

For the mitochondrial alignment, we estimated the phylogenetic relationships among unique haplotypes of *Eidolon* and our outgroup specimens under both maximum likelihood (ML) and Bayesian frameworks. We parameterized maximum likelihood tree searches in RAxML v7.04 (Stamatakis, 2006) using the GTR+Γ model, after partitioning the alignment into two partitions (codons 1 + 2; codon 3) to account for non-synonymous and synonymous substitutions. Tree searches proceeded after 1,000 rapid bootstraps that began with a parsimony guide tree, and followed the default hill-climbing algorithm. For the Bayesian tree searches, we first used MrModeltest2 (Nylander, 2004) to estimate the best-fitting models of sequence evolution at each codon position under the Akaike information criterion. The models HKY+I+Γ, HKY+I, and GTR+Γ were applied to the first, second, and third codon positions respectively, in MrBayes v3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Searches consisted of two independent runs of 10 million generations sampled every 1,000 steps. We checked for adequate mixing within runs and convergence across runs in Tracer v1.6 (Rambaut *et al*., 2013) and summarized the posterior distribution of trees on the half-compatible consensus tree in MrBayes, after discarding the first 25% of samples as burnin.

Divergence Time Estimation

We estimated the number of synonymous and non-synonymous sites at cyt-*b* using DnaSP v5 (Librado and Rozas, 2009) and calculated a rough estimate of substitution rate using averaged mammalian rates of $1.8 \times 10^{-9} \pm 0.3 \times 10^{-9}$ sub/site/yr for non-synonymous sites and $27.4 \times 10^{-9} \pm 3.3 \times 10^{-9}$ sub/site/yr for synonymous sites (Pesole *et al*., 1999). The average number of substitutions with Jukes-Cantor correction within each species and between the two species was also estimated in DnaSP.

We also estimated the divergence time between *E. helvum* and *E. dupreanum* using a coalescent gene tree approach in *BEAST v1.8.0 (Heled and Drummond, 2010; Drummond *et al*., 2012) that included all three gene regions. Cyt-*b* alignments were split into two partitions (codons 1 + 2; codon 3) each assuming a GTR+Γ substitution model. We used an HKY model for *β-fib* and a GTR+Γ model for *RAG1-RAG2*. The prior for the uncorrelated log-normal relaxed clock mean rate of cyt-*b* was 8.23×10^{-9} substitutions/site/yr with an SD of 2.5×10^{-9}. Because we did not have any prior information on rates at *β-fib* or *RAG1-RAG2*, we used flat priors with starting values of 1×10^{-10} subs/site/yr and ranges of 10^{-14} to 10^{-8}. We assumed a Yule species prior and lognormal relaxed clocks for each locus (Drummond *et al*., 2006). The final run was 300 million generations sampled every 10,000 steps. Trees were summarized in TreeAnnotator (Drummond *et al*., 2012) after discarding the first 25% as burnin.

Finally, we estimated the historical population dynamics within each species using multilocus extended Bayesian skyline plots (EBSP) in BEAST v1.8.0. We first estimated the best-fit substitution models at each locus for each species in DT-ModSel (Minin *et al*., 2003). For *E. helvum*, we applied HKY+I, F81, and K80+I substitution models to the cyt-*b, β-fib*, and *RAG1-RAG2* partitions. We applied the same models for the cyt-*b* and *β-fib* partitions to our analysis of *E. dupreanum*, but instead used a Jukes-Cantor model for the *RAG1-RAG2* partition. Final runs were 100 million steps long sampled every 10,000 steps.

Results

For cyt-*b* (1,116 base pairs [bp]), we recovered 37 sequences of *Eidolon helvum*, 80 sequences of *E. dupreanum*, and two sequences each of the *Pteropus* and *Rousettus* outgroups. For *β-fib* (683 bp), *RAG1* (1,054 bp), and *RAG2* (731 bp), we recovered the targeted subset of 26 (25 for *β-fib*) sequences for *E. helvum* and 14 sequences for *E. dupreanum*. For *E. dupreanum*, we recovered 39, two, and two unique haplotypes at cyt-*b*, *β-fib*, and *RAG1-RAG2*, respectively. For *E. helvum*, we recovered 35, three, and five unique haplotypes at the same loci, respectively. All new sequences were deposited in GenBank (Appendix).

For *E. dupreanum*, the final cyt-*b* alignment contained 66 variable sites, 34 of which were parsimony informative. At *β-fib* and *RAG1-RAG2*, there were only two and three variable sites, respectively, none of which were parsimony informative. In *E. helvum*, the final cyt-*b* alignment contained 73 variable sites, of which 30 were parsimony informative. At *β-fib*, there were four variable sites, with one parsimony informative site. At *RAG1-RAG2*, there were 10 variable sites, with five parsimony informative sites.

Haplotype networks for *E. dupreanum* on Madagascar did not support population structure (Fig. 2). Substantial haplotype diversity was observed at

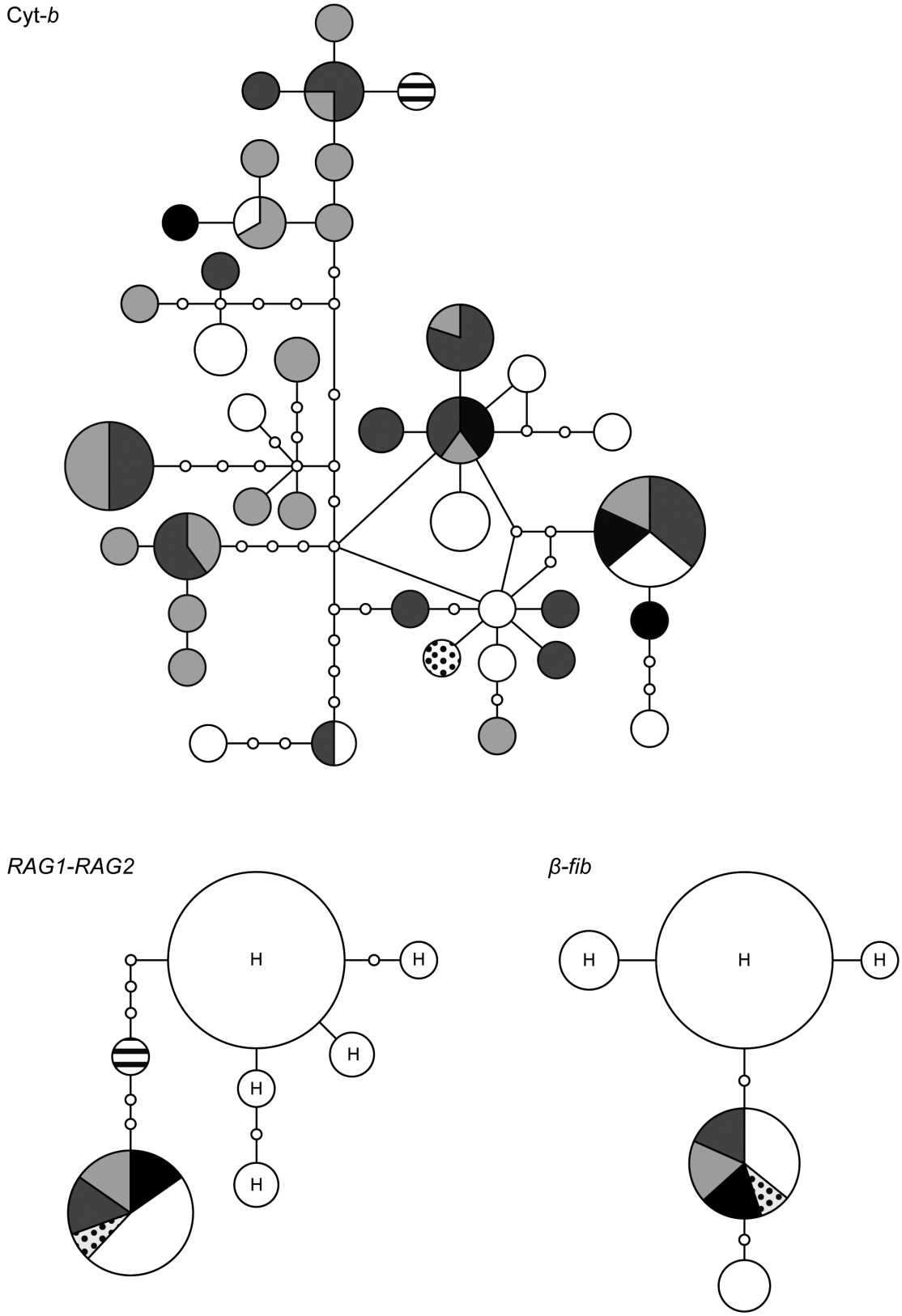

FIG. 2. Haplotype networks for (A) cyt-*b* in *E. dupreanum*, and (B) *β-fib* and concatenated (C) *RAG1-RAG2* in *E. dupreanum* and *E. helvum*. Sizes of circles are proportional to the haplotype frequency within each species. Lines connect haplotypes that differ by one mutational step and small white circles represent inferred, unsampled haplotypes. All *E. helvum* haplotypes are from continental Africa and are indicated with an 'H'. The remaining haplotypes are from *E. dupreanum* and the shades and fill patterns correspond to sampling localities on Madagascar in Fig. 1

cyt-*b* (Fig. 2A). There were numerous shared haplotypes across sampling localities at this locus, resulting in no clear pattern of clustering by locality. Insufficient haplotype diversity was present at *β-fib* and *RAG1-RAG2* to make strong inferences regarding population structure. We found no shared haplotypes between *E. dupreanum* and *E. helvum* for either of these loci, with both species having haplotypes that solely clustered intraspecifically, at opposite ends of the haplotype network (Fig. 2B and 2C). Overall, with either multiple shared haplotypes (cyt-*b*) or a few widespread haplotypes (*β-fib* and *RAG1-RAG2*) that were spread across sampling localities, we found little evidence for geographic structuring in *E. dupreanum*.

Maximum likelihood and Bayesian phylogenetic analyses were congruent regarding node support at the genus and species level (Fig. 3). We recovered high bootstrap (BS) support and posterior probabilities (PP) for the monophyly of the genus *Eidolon* (Fig. 3A) and the monophyly of *E. dupreanum* (Fig. 3C). We did not recover *E. helvum* as a strongly supported monophyletic group (Fig. 3B, BS = 0.560 and PP = 0.538) in phylogenetic analyses of cyt-*b*.

We estimated 25.1% synonymous sites and 74.9% non-synonymous sites among the *Eidolon* samples for an overall mean substitution rate of 8.23×10^{-9} substitutions/site/year. Jukes-Cantor corrected mean sequence divergence at cyt-*b* was low within species (*E. helvum*: 0.834% and *E. dupreanum*: 0.669%) but high between species (11.38%). The mean estimated clade ages of *E. helvum* and *E. dupreanum* were 0.51 Ma and 0.41 Ma, respectively, and the mean estimated divergence time between the species was 6.91 Ma, in the Upper Miocene.

In contrast, the estimates of clade ages and the multilocus estimate of divergence time were greater in multilocus coalescent-based analyses using *BEAST. When we constrained each species to be monophyletic, the median clade age of *E. helvum* was 1.27 Ma (95% HPD: 0.56–2.47 Ma) and that of *E. dupreanum* was 0.89 Ma (95% HPD: 0.41–1.62 Ma). The median estimated divergence time between the species was 11.73 Ma (95% HPD: 7.94–13.44 Ma), in the late Middle Miocene. Extended Bayesian skyline plots for each species estimated that historical population sizes were smaller than current population sizes, though in both instances, the credible intervals around more recent estimates were broad (Fig. 4).

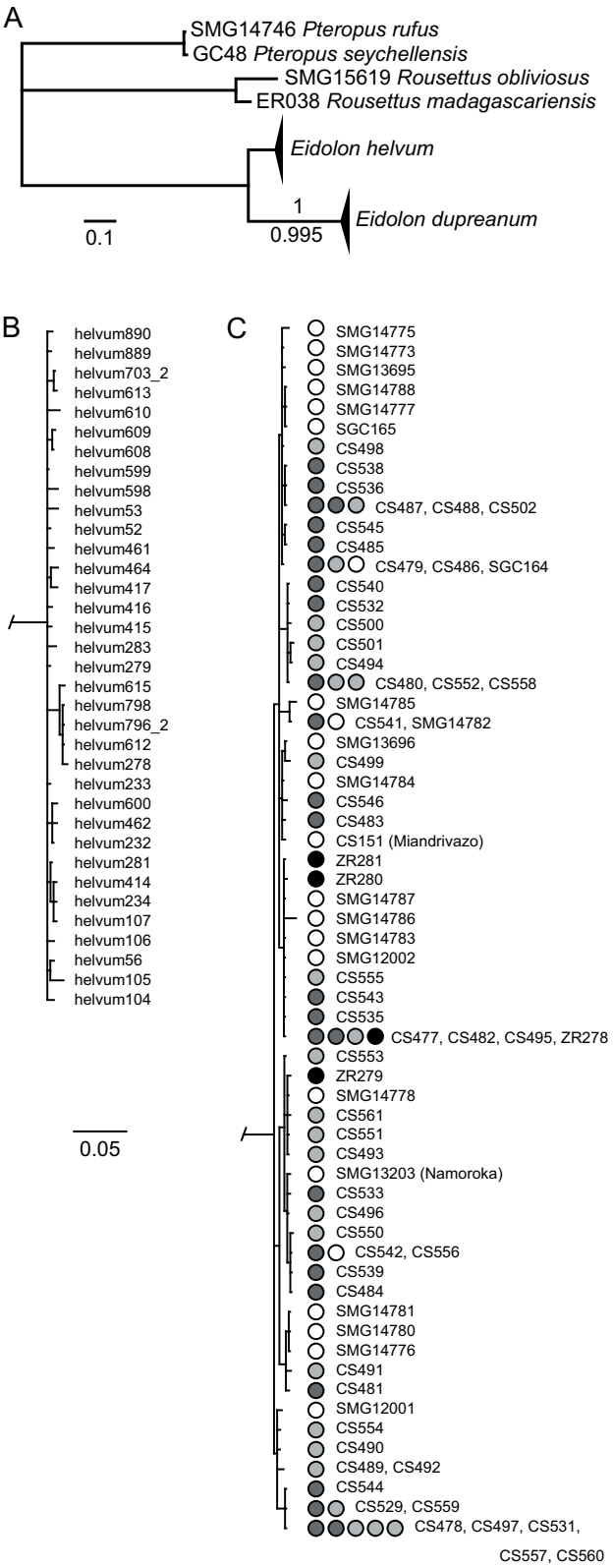

FIG. 3. Bayesian phylogeny pteropodid taxa included in this study (A) with detailed phylogenetic relationships among *E. helvum* (B) and *E. dupreanum* (C). Support values in (A) denote ML bootstrap support (top) and posterior probabilities (bottom) solely at the *E. dupreanum* bipartition, as support for *E. helvum* is less than 0.95. Tip shades and fill patterns in (C) correspond to sampling localities on Madagascar in Fig. 1

Discussion

The divergence time between *Eidolon helvum* and *E. dupreanum* we present here is based on a general substitution rate for mammals, but provides insight on the relative timing of speciation and divergence events. The only known fossil of *Eidolon* is from Ethiopia and is dated to the late Pliocene (3 Ma — Howell and Coppens, 1974). Though our two estimates of divergence time differ and the credible intervals around the Bayesian estimate are broad, both estimated Miocene dates are congruent with previously suggested evolutionary hypotheses (Juste *et al*., 1999, 2000), as well as divergence time estimates from a more recent study (Peel, 2012).

Eidolon helvum is known to migrate long distances on the African continent (+2,500 km over five months), with shorter nightly movements of up to 370 km (Richter and Cumming, 2008). Long-distance migration across open water has also been reported (570 km — Jiménez and Hazevoet, 2010), though it is likely a rare occurrence considering the low population connectivity found among west African oceanic islands separated by only 150–350 km (Juste *et al*., 2000; Peel *et al*., 2013). The Mozambique Channel separating mainland Africa from Madagascar is approximately 460 km at its narrowest point. Given this large distance, we hypothesize that trans-channel dispersal limitations in *Eidolon* are responsible for vicariant speciation between continental and Malagasy species.

Although *E. dupreanum* and *E. helvum* are distinct morphologically, genetically, and geographically, support for the monophyly of *E. helvum* is weak in both ML and Bayesian analyses of mitochondrial sequence data. If *E. dupreanum* diverged recently through colonization of Madagascar from an African relative, as has been previously inferred (Bergmans, 1990), it is not surprising that *E. helvum* might demonstrate paraphyly at individual genetic loci. Recent population expansion from an ancestral gene pool and slow lineage sorting associated with large effective population sizes could contribute to such a pattern in *E. helvum*. It is important to note that these conclusions are based on a single mitochondrial gene. However, multilocus estimates of historical population sizes through time do suggest a rapid demographic expansion in *E. helvum* within the last 0.5 million years (Fig. 4), particularly in comparison to a much more modest increase in *E. dupreanum* population size. The samples available for this study were from a limited number of localities (Fig. 1, Appendix), and finer geographic sampling of individuals from both species and additional genetic loci may help resolve the underlying evolutionary and demographic scenario characterizing the divergence of these two taxa.

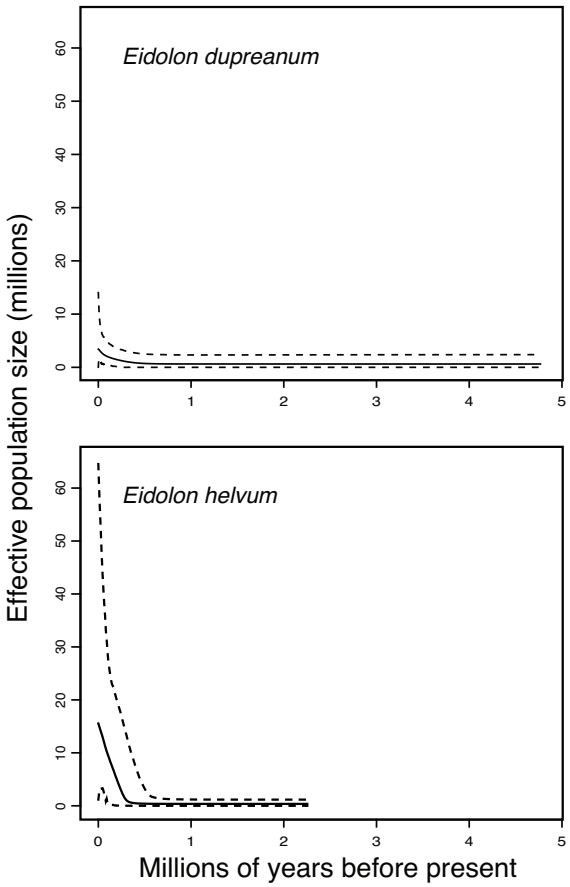

Fig. 4. Extended Bayesian skyline plots of estimated historical effective population sizes for *E. helvum* and *E. dupreanum* based on cyt-*b*, *β-fib*, and concatenated *RAG1-RAG2*. Dotted lines denote 95% credible intervals

Despite the pattern of deep divergence between these two species, we find high population connectivity in *E. dupreanum* across Madagascar, similar to that found in *E. helvum* across continental Africa (Peel *et al*., 2013). For both taxa, high vagility has resulted in little to no population diversification below the species level (Fig. 2). This pattern of low population structure exists across both species despite notable interspecific differences in the placement of day roost sites and the number of roosting bats. In *E. helvum*, roosts can be extremely large, reaching more than one million individuals, and are typically found in trees across a considerable range of vegetation types and in urban areas (Nowak and Roland, 1999; Sørensen and Halberg, 2001). In contrast, roost sites for *E. dupreanum*

are almost strictly in rock-crevasses or caves, and colonies rarely reach more than 1000 individuals (MacKinnon *et al*., 2003; Goodman, 2011). There appears to have been little historic variation in roost sites in recent geological time (Burney *et al*., 2008), as well, though modern anthropogenic disturbance may be affecting roost density and site abandon-ment (Cardiff *et al*., 2009; Goodman and Jungers, 2014). At least at the resolution of these genetic markers, differences between the two species in colony size and roosting habitats do not lead to interspecific variation in population genetic connectivity.

Aspects of feeding ecology might explain regular dispersal movements of the frugivorous *E. dupreanum* (Picot *et al*., 2007; Ratrimomanarivo, 2007). Phenological patterns of fruiting in different forest formations on Madagascar are notably seasonal, and periods exist when fruits are limited (Goodman and Ganzhorn, 1997). Frugivorous animals, including bats, must either seasonally shift to different diets or disperse to areas where fruit resources are available (Andriafidison *et al*., 2006). Maximum entropy distribution modeling (MaxEnt) incorporating aspects of this species' distribution based on 78 geographical points and an assortment of environmental variables has found that geology is an important explanatory variable of their occurrence (Goodman and Ramasindrazana, 2013). The reliance of *E. dupreanum* on broadly dispersed habitats like rocky outcrops and cave roosts, coupled with seasonal patterns of food availability, can help explain the demonstrated patterns of genetic panmixia. Virtually nothing is known about the reproductive biology and mating habits of *E. dupreanum*, which could also be linked to dispersal patterns of this species.

Though low levels of population structure are typically expected for vagile mammals, including bats (Burland and Worthington-Wilmer, 2001), ongoing research has highlighted significant variability in population structure depending on the life history traits of the species in question (Petit and Mayer, 2000; Rivers *et al*., 2005; Russell *et al*., 2005). In addition, connectivity and genetic structure are dependent upon the complex relationships among the competing factors that isolate incipient species and the factors that precipitate long-distance dispersal and migration (Dynesius and Jansson, 2013). Here, we demonstrate that despite extensive population connectivity within each species, *E. dupreanum* and *E. helvum* individually represent old, persistent, and distinct lineages.

Characterizing the nature of migration patterns and population connectivity is important not only for our understanding of species divergence, but also for inferences associated with zoonotic pathogens. Bat populations, particularly fruit bats of the family Pteropodidae, are reservoirs for numerous pathogens, a number of which can be transmitted to humans (e.g., Reynes *et al*., 2005; Wong *et al*., 2007). Understanding how population connectivity relates to transmission dynamics in pteropodid bats like *Eidolon* is thus of particular interest because these species can live in high densities in close proximity to humans and are often consumed as bushmeat (Jenkins and Racey, 2008; Goodman, 2011; Peel *et al*., 2013).

Recent work has found that *E. helvum* acts as a reservoir host for *Lyssavirus* (Lagos bat virus) and henipaviruses across its widespread range, and continental-scale viral transmission within *E. helvum* is likely facilitated by its panmictic population structure (Peel *et al*., 2013). Given our results regarding the connectivity of *E. dupreanum* and its interactions with humans on Madagascar, it seems likely that this species is also of epidemiological importance (Andriamandimby *et al*., 2013). Combining phylogeographic patterns within *Eidolon* with a better understanding of its evolutionary history will provide the means to pose explicit hypotheses regarding the introduction of pathogens from initial colonization and/or lateral transfer from other vectors. Future research should sample more broadly across Madagascar, to understand the potential implications of *E. dupreanum* as a vector for human disease on the island.

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Appendix

Eidolon helvum and *E. dupreanum* samples used for this study, including field number, museum catalog number/voucher identification, sex, collection locality, collection coordinates, and GenBank accession numbers for each locus sequenced. n/a = not applicable in the case of museum catalog number as specimen was not collected. Abbreviations: FMNH — Field Museum of Natural History, Chicago, Illinois, USA; UADBA — Université d'Antananarivo, Département de Biologie Animale, Antananarivo, Madagascar; P — Zoology Department of Makerere University, Kampala, Uganda

Taxon	Field number	Voucher ID	Sex	Locality	Latitude	Longitude	cyt-*b*	*ß-fib*	*RAG1*	*RAG2*
E. dupreanum	CS 151	n/a	male	Madagascar: Miandrivazo	19.522222°S	45.457222°E	KM225999	KM225948	KM226079	KM226119
E. dupreanum	CS 477	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226000	KM225949	KM226080	KM226120
E. dupreanum	CS 478	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226001	KM225950	KM226081	KM226121
E. dupreanum	CS 479	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226002			
E. dupreanum	CS 480	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226003			
E. dupreanum	CS 481	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226004			
E. dupreanum	CS 482	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226005			
E. dupreanum	CS 483	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226006			
E. dupreanum	CS 484	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226007			
E. dupreanum	CS 485	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226008			
E. dupreanum	CS 486	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226009			
E. dupreanum	CS 487	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226010			
E. dupreanum	CS 488	n/a	unknown	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226011			
E. dupreanum	CS 489	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226012	KM225951	KM226082	KM226122
E. dupreanum	CS 490	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226013	KM225952	KM226083	KM226123
E. dupreanum	CS 491	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226014			
E. dupreanum	CS 492	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226015			
E. dupreanum	CS 493	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226016			
E. dupreanum	CS 494	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226017			
E. dupreanum	CS 495	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226018			
E. dupreanum	CS 496	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226019			
E. dupreanum	CS 497	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226020			
E. dupreanum	CS 498	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226021			
E. dupreanum	CS 499	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226022			
E. dupreanum	CS 500	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226023			
E. dupreanum	CS 501	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226024			
E. dupreanum	CS 502	n/a	unknown	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226025			
E. dupreanum	CS 529	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226026			
E. dupreanum	CS 531	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226027			
E. dupreanum	CS 532	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226028			
E. dupreanum	CS 533	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226029			
E. dupreanum	CS 535	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226030			
E. dupreanum	CS 536	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226031			
E. dupreanum	CS 538	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226032			
E. dupreanum	CS 539	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226033			
E. dupreanum	CS 540	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226034			
E. dupreanum	CS 541	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226035			
E. dupreanum	CS 542	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226036			
E. dupreanum	CS 543	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226037			
E. dupreanum	CS 544	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226038			
E. dupreanum	CS 545	n/a	female	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226039			
E. dupreanum	CS 546	n/a	male	Madagascar: Carion, Angavokely	18.927500°S	47.753611°E	KM226040			
E. dupreanum	CS 550	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226041			

APPENDIX. Continued

Taxon	Field number	Voucher ID	Sex	Locality	Latitude	Longitude	cyt-*b*	*ß-fib*	*RAG1*	*RAG2*
E. dupreanum	CS 551	n/a	male	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226042			
E. dupreanum	CS 552	n/a	male	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226043			
E. dupreanum	CS 553	n/a	male	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226044			
E. dupreanum	CS 554	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226045			
E. dupreanum	CS 555	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226046			
E. dupreanum	CS 556	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226047			
E. dupreanum	CS 557	n/a	male	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226048			
E. dupreanum	CS 558	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226049			
E. dupreanum	CS 559	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226050			
E. dupreanum	CS 560	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226051			
E. dupreanum	CS 561	n/a	female	Madagascar: Marozevo, Falaise Angavobe	18.918056°S	47.943611°E	KM226052			
E. dupreanum	SGC 164	FMNH 177381	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe, Grotte Andokotokana	12.943610°S	49.0547°E	KM226053	KM225953	KM226084	KM226124
E. dupreanum	SGC 165	FMNH 177382	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe, Grotte Andokotokana	12.943610°S	49.0547°E	KM226054	KM225954	KM226085	KM226125
E. dupreanum	SMG 12001	FMNH 169701	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe, Grotte Andokotokana	12.941666°S	49.055°E	KM226055	KM225955	KM226086	KM226126
E. dupreanum	SMG 12002	FMNH 169702	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe, Grotte Andokotokana	12.941666°S	49.055°E	KM226056	KM225956	KM226087	KM226127
E. dupreanum	SMG 13203	FMNH 175757	male	Madagascar: RNI de Namoroka, Grotte d'Ankopimpanihy	16.406666°S	45.31°E	KM226057	KM225957	KM226088	KM226128
E. dupreanum	SMG 13695	FMNH 176261	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.941666°S	49.055°E	KM226058			
E. dupreanum	SMG 13696	FMNH 176358	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.941666°S	49.055°E	KM226059			
E. dupreanum	SMG 14773	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226060			
E. dupreanum	SMG 14775	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226061	KM225958	KM226089	KM226129
E. dupreanum	SMG 14776	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226062	KM225959	KM226090	KM226130
E. dupreanum	SMG 14777	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226063			
E. dupreanum	SMG 14778	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226064			
E. dupreanum	SMG 14779	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226065			
E. dupreanum	SMG 14780	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226066			
E. dupreanum	SMG 14781	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226067			
E. dupreanum	SMG 14782	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226068			
E. dupreanum	SMG 14783	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226069			
E. dupreanum	SMG 14784	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226070			
E. dupreanum	SMG 14785	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226071			
E. dupreanum	SMG 14786	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226072			
E. dupreanum	SMG 14787	n/a	male	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226073			
E. dupreanum	SMG 14788	n/a	female	Madagascar: RS d'Ankarana, 3.5 km SE Andrafiabe (village)	12.943610°S	49.0547°E	KM226074			
E. dupreanum	ZR 278	FMNH 194642	male	Madagascar: District de Morombe, Nosy Ambositra, Antevankira Forest, 17 km ESE Ambiky	21.945833°S	44.046389°E	KM226075	KM225960	KM226091	KM226131
E. dupreanum	ZR 279	FMNH 194643	female	Madagascar: District de Morombe, Nosy Ambositra, Antevankira Forest, 17 km ESE Ambiky	21.945833°S	44.046389°E	KM226076	KM225961	KM226092	KM226132
E. dupreanum	ZR 280	UADBA 48290	female	Madagascar: District de Morombe, Nosy Ambositra, Antevankira Forest, 17 km ESE Ambiky	21.945833°S	44.046389°E	KM226077			
E. dupreanum	ZR 281	UADBA 48289	female	Madagascar: District de Morombe, Nosy Ambositra, Antevankira Forest, 17 km ESE Ambiky	21.945833°S	44.046389°E	KM226078			

APPENDIX. Continued

Taxon	Field number	Voucher ID	Sex	Locality	Latitude	Longitude	cyt-*b*	*ß-fib*	*RAG1*	*RAG2*
E. helvum	helvum104 (DRC-104)	n/a	male	Democratic Republic of the Congo: Kisangani	0.52800°N	25.37100°E	KM225962	KM225923	KM226093	KM226133
E. helvum	helvum105 (DRC-105)	n/a	female	Democratic Republic of the Congo: Kisangani	0.52800°N	25.37200°E	KM225963	KM225924	KM226094	KM226134
E. helvum	helvum106 (DRC-106)	n/a	male	Democratic Republic of the Congo: Kisangani	0.52800°N	25.37300°E	KM225964			
E. helvum	helvum107 (DRC-107)	n/a	female	Democratic Republic of the Congo: Kisangani	0.52800°N	25.37400°E	KM225965			
E. helvum	helvum232 (K001)	n/a	male	Zambia: Kasanka National Park	12.58862°S	30.24623°E	KM225966	KM225925	KM226095	KM226135
E. helvum	helvum233 (K002)	n/a	female	Zambia: Kasanka National Park	12.58862°S	30.24623°E	KM225967	KM225926	KM226096	KM226136
E. helvum	helvum234 (K003)	n/a	female	Zambia: Kasanka National Park	12.58862°S	30.24623°E	KM225968			
E. helvum	helvum278 (K047)	n/a	male	Malawi: Blantyre	15.78841°S	35.01051°E	KM225969		KM226097	KM226137
E. helvum	helvum279 (K048)	n/a	female	Malawi: Blantyre	15.78841°S	35.01051°E	KM225970	KM225927	KM226098	KM226138
E. helvum	helvum281 (K050)	n/a	female	Malawi: Blantyre	15.78841°S	35.01051°E	KM225971			
E. helvum	helvum283 (K052)	n/a	female	Malawi: Blantyre	15.78841°S	35.01051°E	KM225972			
E. helvum	helvum414 (RM-126)	n/a	unknown	Equatorial Guinea: Rio Muni	exact coordinates unavailable		KM225973	KM225928	KM226099	KM226139
E. helvum	helvum415 (RM-127)	n/a	unknown	Equatorial Guinea: Rio Muni	exact coordinates unavailable		KM225974	KM225929	KM226100	KM226140
E. helvum	helvum416 (RM-128)	n/a	unknown	Equatorial Guinea: Rio Muni	exact coordinates unavailable		KM225975			
E. helvum	helvum417 (RM-129)	n/a	unknown	Equatorial Guinea: Rio Muni	exact coordinates unavailable		KM225976			
E. helvum	helvum461 (T002)	n/a	female	Tanzania: Dar es Salaam, Lugalo Rd,	6.80134°S	39.28249°E	KM225977	KM225930	KM226101	KM226141
E. helvum	helvum462 (T003)	n/a	male	Tanzania: Dar es Salaam, Lugalo Rd,	6.80134°S	39.28249°E	KM225978	KM225931	KM226102	KM226142
E. helvum	helvum464 (T005)	n/a	female	Tanzania: Dar es Salaam, Lugalo Rd,	6.80134°S	39.28249°E	KM225979			
E. helvum	helvum52 (E52)	n/a	male	Ghana: Accra, 37 Hospital	5.58820°N	0.18239°W	KM225980	KM225932	KM226103	KM226143
E. helvum	helvum53 (E53)	n/a	male	Ghana: Accra, 37 Hospital	5.58820°N	0.18239°W	KM225981	KM225933	KM226104	KM226144
E. helvum	helvum56 (E56)	n/a	male	Ghana: Accra, 37 Hospital	5.58820°N	0.18239°W	KM225982			
E. helvum	helvum598 (T178)	n/a	female	Uganda: Jinja, Bridge Rd	0.42669°N	33.20086°E	KM225983	KM225934	KM226105	KM226145
E. helvum	helvum599 (T180)	n/a	male	Uganda: Jinja, Tirangle Hotel	0.41581°N	33.20388°E	KM225984	KM225935	KM226106	KM226146
E. helvum	helvum600 (T181)	P875	female	Uganda: Kampala, Wandeyega (Bat Valley)	~ 0.33°N	32.57°E	KM225985			
E. helvum	helvum608 (T189)	n/a	male	Uganda: Kampala, Rubaga	0.30305°N	32.55412°E	KM225986	KM225936	KM226107	KM226147
E. helvum	helvum609 (T190)	n/a	male	Uganda: Kampala, Rubaga	0.30305°N	32.55412°E	KM225987			
E. helvum	helvum610 (GoG-1)	n/a	male	São Tomé and Príncipe: São Tomé, Cruzeiro	0.28612°N	6.67806°E	KM225988	KM225937	KM226108	KM226148
E. helvum	helvum612 (GoG-3)	n/a	female	São Tomé and Príncipe: São Tomé, Cruzeiro	0.28612°N	6.67806°E	KM225989	KM225938	KM226109	KM226149
E. helvum	helvum613 (GoG-4)	n/a	male	São Tomé and Príncipe: São Tomé, Roça Nova	0.25232°N	6.67064°E	KM225990	KM225939	KM226110	KM226150
E. helvum	helvum614 (GoG-5)	n/a	male	São Tomé and Príncipe: São Tomé, Canecão	0.34063°N	6.56286°E		KM225940	KM226111	KM226151
E. helvum	helvum615 (GoG-6)	n/a	male	São Tomé and Príncipe: São Tomé, Canecão	0.34063°N	6.56286°E	KM225991	KM225941	KM226112	KM226152
E. helvum	helvum703 (GoG-96)	n/a	female	São Tomé and Príncipe: São Tomé, Porto Alegre	0.02887°N	6.53198°E	KM225992	KM225942	KM226113	KM226153
E. helvum	helvum704 (GoG-97)	n/a	female	São Tomé and Príncipe: São Tomé, Porto Alegre	0.02887°N	6.53198°E	KM225993	KM225943	KM226114	KM226154
E. helvum	helvum796 (GoG-323)	n/a	male	Equatorial Guinea: Annobón, Mabana	1.45918°S	5.64530°E	KM225994	KM225944	KM226115	KM226155
E. helvum	helvum797 (GoG-324)	n/a	male	Equatorial Guinea: Annobón, Mabana	1.45918°S	5.64530°E	KM225995	KM225945	KM226116	KM226156
E. helvum	helvum798 (GoG-325)	n/a	male	Equatorial Guinea: Annobón, Mabana	1.45918°S	5.64530°E	KM225996			
E. helvum	helvum889 (GoG-441)	n/a	female	Equatorial Guinea: Bioko, Malabo	3.75211°N	8.77230°E	KM225997	KM225946	KM226117	KM226157
E. helvum	helvum890 (GoG-442)	n/a	female	Equatorial Guinea: Bioko, Malabo	3.75211°N	8.77230°E	KM225998	KM225947	KM226118	KM226158